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Linkage of Congenital Isolated Adrenocorticotropic Hormone Deficiency to the Corticotropin Releasing Hormone Locus Using Simple Sequence Repeat Polymorphisms

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Genetic screening techniques using simple sequence repeat polymorphisms were applied to investigate the molecular nature of congenital isolated adrenocorticotropic hormone (ACTH) deficiency. We hypothesize that this rare cause of hypocortisolism shared by a brother and sister with two unaffected sibs and unaffected parents is inherited as an autosomal recessive single gene mutation. Genes involved in the hypothalamic-pituitary axis controlling cortisol sufficiency were investigated for a causal role in this disorder. Southern blotting showed no detectable mutations of the gene encoding pro-opiomelanocortin (*POMC*), the ACTH precursor. Other candidate genes subsequently considered were those encoding neuroendocrine convertase-1, and neuroendocrine convertase-2 (*NEC-1*, *NEC-2*), and corticotropin releasing hormone (*CRH*). Tests for linkage were performed using polymorphic di- and tetranucleotide simple sequence repeat markers flanking the reported map locations for *POMC*, *NEC-1*, *NEC-2*, and *CRH*. The chromosomal haplotypes determined by the markers flanking the loci for *POMC*, *NEC-1*, and *NEC-2* were not compatible with linkage. However, 22 individual markers defining the chromosomal haplotypes flanking *CRH* were compatible with linkage of the disorder to the immediate area of this gene on chromosome 8. Based on these data, we hypothesize that the ACTH deficiency in this family is due to an abnormality of CRH gene structure or expression. These results illustrate the useful application of high density genetic maps constructed with simple sequence repeat markers for inclusion/exclusion studies of candidate genes in even very small nuclear families segregating for unusual phenotypes. © 1996 Wiley-Liss, Inc.

KEY WORDS: adrenocorticotropic hormone, corticotropin releasing hormone, simple sequence repeat markers, linkage analysis

INTRODUCTION

Isolated congenital hypocortisolism is an uncommon disorder. Except for cases of steroid biosynthetic defects, most cases of clinical deficiency of cortisol in the neonatal period are associated with overwhelming infections or hypotensive episodes with subsequent damage to the adrenal glands. Rare cases of congenital adrenal hypoplasia have been described, either sporadic or X-linked, and are generally associated with abnormal size or histologic structure of the adrenal gland [Migeon and Donohoue, 1994; Bartley et al., 1986; Muscatelli et al., 1994]. Other cases of congenital adrenal insufficiency, generally concurrent with other pituitary hormone deficiencies, have been associated with hypoplasia of the pituitary gland or with gross brain anomalies such as anencephaly. There have also been a number of case reports describing congenital ACTH deficiency with the defect attributed to ACTH itself, deficient activity of one of the neuroendocrine convertases, or with the cause not specifically identified beyond being a secondary adrenal insufficiency

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[Malpuech et al., 1988; Ichiba and Goto, 1983; Carey, 1985; Nussey et al., 1993].

We have studied a brother and sister, from a non-consanguineous marriage who have congenital cortisol insufficiency and laboratory evidence of ACTH deficiency. Pituitary imaging was normal, but an asymptomatic Chiari I malformation was present in both. This family, comprised of the two affected children, two unaffected children, and unaffected parents, presented an optimal situation to apply newer modes of genetic screening techniques as a first step towards investigating the molecular mechanisms of a clinical syndrome.

From the clinical information, we hypothesized that the hypocortisolism in these sibs resulted from an autosomal recessive disorder due to a single gene defect. This cortisol deficiency could result from any of a number of hormone defects. However, based on clinical and laboratory data, notably, measured values of ACTH and cortisol being very low with normal electrolytes, the defect appeared to be due to a deficiency of ACTH or CRH, rather than resistance to ACTH. Four candidate genes were chosen for study: the genes encoding pro-opiomelanocortin (*POMC*), the precursor for ACTH; the two neuroendocrine convertases (*NEC-1,* and *NEC-2*), which are necessary for processing the POMC gene product to ACTH [Zhou et al., 1993], and corticotropin releasing hormone (*CRH*). We used both restriction fragment length polymorphism (RFLP) and simple sequence repeat polymorphisms for detection of gene rearrangements and linkage analysis [Jeffreys et al., 1985; Weber, 1990]. This method allowed us to screen our candidate genes in order to more rapidly exclude unlinked candidates prior to expending greater effort examining the potentially linked genes more closely. Useful information may be obtained in a small family such as this, as the high density and heterozygosity of simple sequence repeat markers provide great power in excluding linkage to candidate genes even in small pedigrees. We utilized markers to assess candidate gene linkage, and chose several which flanked each candidate gene in question.

MATERIALS AND METHODS

Clinical Histories

The older sib (sister) was diagnosed at age 2 months when she presented with pneumonia, seizures and hypoglycemia with a blood glucose of 18 mg/dl. Laboratory studies at that time showed undetectable levels of cortisol (AM and PM). Insulin was <5 mcU/dl, T4 was 9.5 mcg/dl, electrolytes were normal, and 24 hour 17-hydroxycorticosteroids were low at 0.7 mg/m^2/24 hours (nl = 2–4). At 2 years of age an arginine stimulation test showed growth hormone levels of 3.1, 4.0, 29.8 and 20.9 ng/ml at 0, 30, 60, and 90 minutes, respectively. The younger brother was diagnosed on the first day of life when his blood glucose dropped to 15 mg/dl. His initial cortisol and ACTH levels were low as well. Following several years of cortisol replacement, a series of pre-prandial ACTH levels in sister and brother were 18–30 pg/ml, and 21–27 pg/ml, respectively (n = 20–100 pg/ml). Two years later, both children developed nausea and lethargy when glucocorticoid replacement was decreased in preparation for testing, so cortisol replacement was discontinued for only 1 day. CRH stimulation tests on both documented low baseline ACTH levels and minimal ACTH response to exogenous CRH stimulation (Table I). Cortisol levels were undetectable for the duration of the test.

Both sibs have had normal growth. The sister is now 13 years old and has shown normal pubertal development including menarche. Both have had normal intellectual development as well. Pituitary sizes were estimated to be normal by MRI imaging; however, both were also found to have asymptomatic Arnold-Chiari type 1 malformations.

Genomic DNA was extracted from EBV transformed blood lymphocytes [Hansen et al., 1979] from each family member. DNA previously obtained in a similar manner from other unrelated, unaffected persons was used as a normal control for all procedures.

Southern Blots

The POMC gene locus was initially examined via restriction digest and hybridization to a POMC-exon 3 specific probe following Southern transfer. Genomic DNA was digested with three combinations of restriction enzymes (EcoRI, EcoRI and BglII, EcoRI, and XhoI) dividing the POMC gene into fragments encompassing one, two or three of the three exons (Fig. 1). This combination of digests would enable assignment of fragment length discrepancies to the exon responsible. The digested DNA was separated on an agarose gel, transferred to a nylon membrane (Zeta Probe, BioRad Laboratories, Hercules, CA), hybridized to an [α^{32}P] dCTP-labeled probe [Feinberg and Vogelstein, 1984]. The probe was a SmaI-SmaI 1.1 kb 3′ fragment including almost all of exon three, and detected by autoradiography. Hybridization and washing conditions were performed as described [Donohoue et al., 1986].

Simple Sequence Repeat Studies

Genomic DNA obtained from patients and controls as described above was used as a template in a series of standard polymerase chain reactions using the following conditions: 95°C × 10 minutes, then 35 cycles of 94° × 30 seconds, 55° × 30 seconds, and 72° × 30 seconds. The oligonucleotide primers for the dinucleotide repeats were purchased (Research Genetics, Huntsville, AL). Choice of markers was based on published reports or on maps [NIH/CEPH Collab. Mapping Group, 1992; Seo et al., 1993; Gu et al., 1993; Murray et al., 1993, 1994]. These polymorphic tandem repeats were amplified us-

TABLE I. CRH Stimulation Test Results for the Sibs With ACTH Deficiency

	ACTH (pg/ml)	
	Basal	60 minutes
Sister	1.8	1.7
Brother	1.65	3.4
Normal range	20–100	

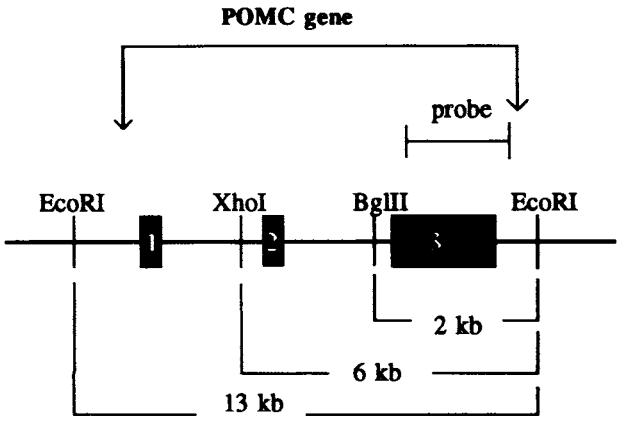

Fig. 1. Representation of a portion of chromosome 2, including the POMC gene. Predicted restriction sites and fragment lengths are noted. Arrows indicate the approximate boundaries of the gene, boxes 1, 2, and 3 indicate the locations of the three exons within this gene. Area of hybridization with the probe used is also indicated. DNA from all relatives showed restriction fragments of the expected lengths as shown above, via Southern analysis.

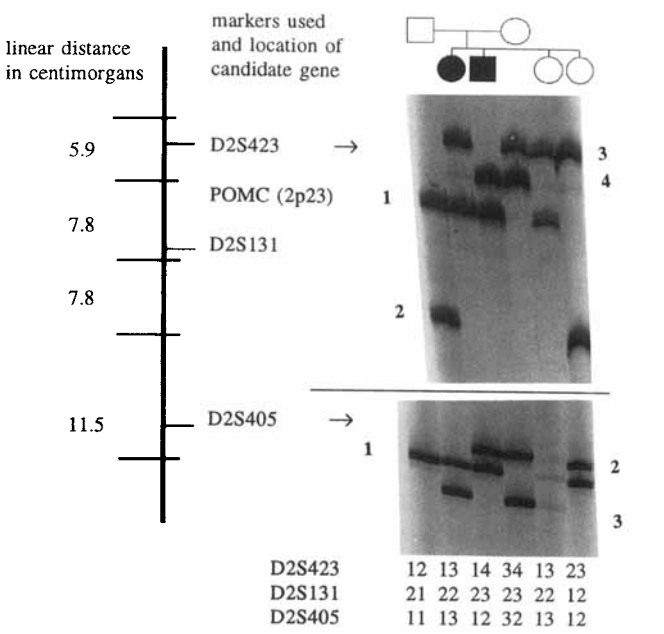

Fig. 2. Microsatellite analysis of a portion of chromosome 2p. The approximate locations of the three tetranucleotide repeat markers relative to the POMC gene are indicated. In all figures: sex-averaged recombination distances delineated by specific markers (horizontal lines) are indicated in cM. Autoradiographs of the amplified markers are shown with the family tree above the lanes for identification; the probands are represented by the filled circle and square. Arbitrary numbers were assigned to each allele size for ease of identification in constructing haplotypes. The alleles comprising each haplotype can be read vertically, from top to bottom, directly beneath each member of the pedigree. These autoradiographs are representative of the results observed for all the other markers used, and are shown in this figure as an example of the raw data. Here, the affected sibs have different paternal haplotypes, and one unaffected sister shares the same maternal and paternal haplotypes as her affected brother.

ing [α-^{35}S]dATP, separated on 5% or 8% denaturing polyacrylamide gels, and detected by autoradiography.

ACTH Precursor Studies

Serum samples were also analyzed for presence of ACTH precursors as a second way to assess the integrity of the *POMC, NEC-1,* and *NEC-2* transcripts. Both affected children were receiving glucocorticoid replacement at the time the blood samples were drawn. Serum was separated, frozen and analyzed for ACTH precursors [Crosby et al., 1988].

RESULTS

Southern Blots

The Southern blots hybridized with a POMC specific probe showed the expected restriction fragment lengths for all relatives and controls. The EcoRI-EcoRI fragment containing all three exons of the gene was 13 kb in all members, the XhoI-EcoRI fragment containing exons 2 and 3 was 6 kb in all members, and the BglII-EcoRI fragment containing exon 3 was 2 kb in all members (Fig. 1). These fragment sizes were the same as observed in several control samples tested as well, suggesting that the POMC locus in members of this family has no large deletions or rearrangements [Kyllo et al., 1993].

Linkage Studies

We assumed autosomal recessive inheritance is the likely mechanism of inheritance given unaffected parents and affected siblings of the opposite sex. We established the genotypes at three tetranucleotide repeat markers on chromosome 2p which flank the gene encoding POMC (Fig. 2). These loci, D2S423, D25131, and D2S405, span approximately 28-33 cM including the POMC locus. At this locus, linkage was likely excluded as the two affected siblings do not share the same alleles, and one unaffected sister shares the same haplotypes as her affected sister.

Figure 3 shows the approximate map locations and products of amplification of four markers (D5S815, D5S1467, D5S1549, and GATA45) on chromosome 5 in proximity to the gene encoding neuroendocrine convertase-1. Linkage could not be assessed based on genotypes of each individual locus, as this family was not fully polymorphic for any of the four. By combining the genotypes of all four markers, we constructed haplotypes for each individual, providing adequate information ruling against linkage. The affected brother and sister have inherited different paternal haplotypes (2,1,1,1 vs. 1,2,1,1), and one unaffected sister shares the same maternal and paternal haplotypes as her affected brother (2,1,1,1 and 3,2,3,3).

Three markers, NEC2, D20S58, and D20S206, were identified near the gene encoding neuroendocrine convertase-2 on chromosome 20p11.2. The NEC2 marker is within the second intron of the gene but is only informative in the mother. And although the exact linkage map based location of NEC2 has not been assigned, it is tightly linked to D20S58 [Seo et al., 1993]. With the information from the three markers used, linkage of the ACTH deficiency to the NEC-2 locus was excluded as

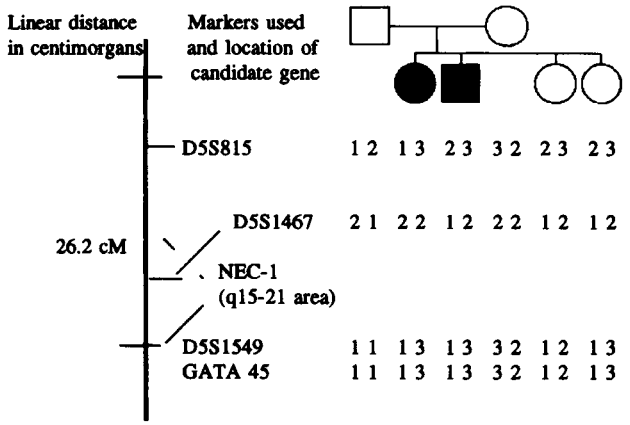

Fig. 3. Microsatellite analysis of the portion of chromosome 5 which includes NEC-1. The approximate locations of the four markers relative to the NEC-1 gene are shown. The genotypes for each family member were labeled as described in Figure 1 and appear in order as shown under the pedigree diagram. The affected sibs have different paternal haplotypes, and one unaffected sister shares the same maternal and paternal haplotypes as her affected brother.

the affected siblings inherited different sets of paternal alleles, and one unaffected sister shares the same parental haplotypes as her affected sister (1,1,1 and 2,2,4) (Fig. 4).

Seven tetranucleotide markers (GATA21, D8S591, D8S587, D8S566,D8S569, D8S594, and D8S1105) which flank the estimated location of the CRH gene on chromosome 8 were initially analyzed. These genotypes were consistent with linkage of the disorder to this location on chromosome 8; however, there was an apparent crossover fairly close to the CRH locus on the maternal haplotype in two of the four sibs (Fig. 5) [Kyllo et al., 1994]. Amplification of a CRH adjacent marker designated "CRH" in the affected family showed minimal polymorphism in that three of the four parental alleles were identical in size. In order to determine the exact locations of the crossovers and thus

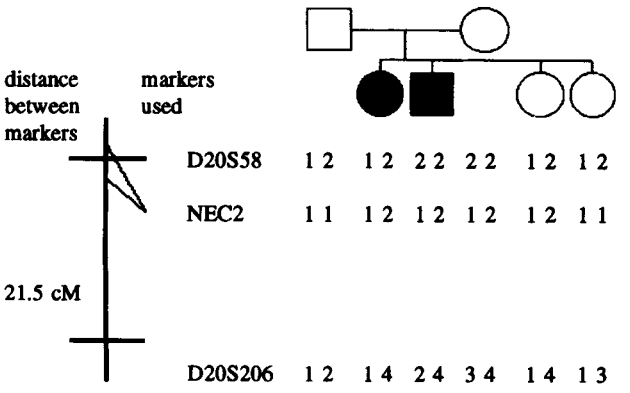

Fig. 4. Microsatellite analysis of chromosome 20. The approximate locations of the three markers used in relation to the NEC-2 gene are shown. The marker designated NEC2 is within one intron of this gene. Genotypes and haplotypes were determined as described above. The affected sibs have inherited different paternal haplotypes and one unaffected sister is haploidentical with her affected sister.

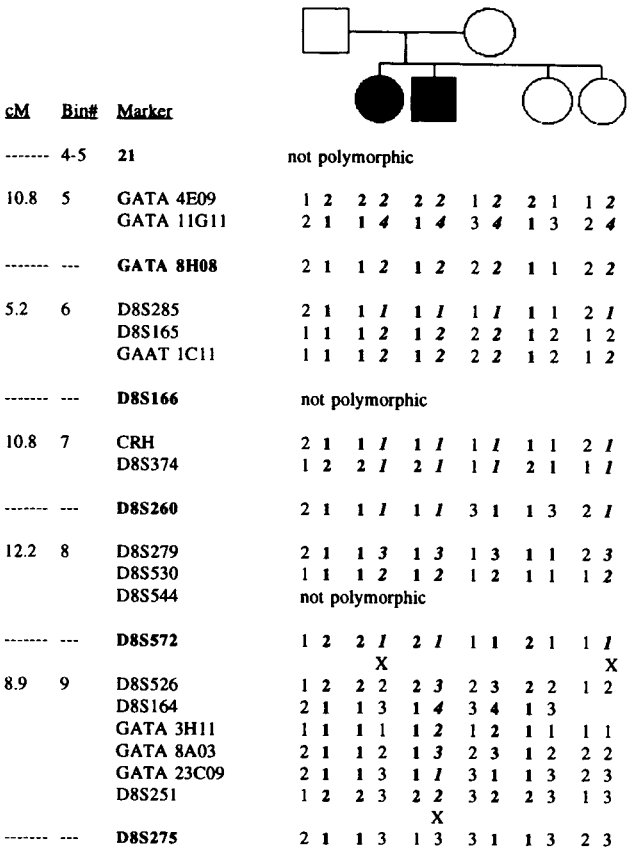

Fig. 5. Microsatellite analysis of chromosome 8q. The markers in bold print are listed in sequence order with centimorgan distances between them indicated on the left. These markers define the limits of "bins" as described in reference 8. The intervening markers listed are known to be within each specific bin, however, the exact order of these has not been determined. The cytogenetic location of the CRH gene is in the 8q13 area, and the recombination based location of the CRH adjacent dinucleotide repeat, and thus CRH gene is placed as shown above. Numeric marker designations typed in bold or italics designate the putative disease carrying paternal and maternal haplotypes, respectively, with Xs representing crossovers.

establish or rule out linkage, genotypes of 14 additional more closely spaced dinucleotide repeats were determined. When the genotypes of these 22 loci were combined to construct haplotypes, the markers showed the two affected sibs inheriting the same maternal and paternal haplotypes, and the unaffected sisters sharing no more than one haplotype with an affected sib. There has been a crossover on the maternal haplotype in two of the four children at least 12.2 cM downstream from the map location of *CRH* (Fig. 5).

ACTH Precursors

Serum samples assayed for ACTH precursors showed normal levels of precursors present in both affected sibs as well as their mother (Table II). Serum samples from other relatives were not assayed for these precursors.

DISCUSSION

We have studied a family which appears to have a single hormone deficiency inherited in an autosomal recessive manner. We have concluded that the diagnosis

TABLE II. Values of ACTH Precursors Measured From Random Serum Samples*

Status	ACTH precursors (pmol/L)
Mother	25
Affected sister	20
Affected brother	23
Normal range	<5–40

*Both affected children were receiving cortisone replacement at the time of the sample draw. The normal adult range is also indicated.

of secondary adrenal insufficiency is correct, based on multiple studies including the random ACTH levels and the CRH stimulation tests. However, the specific level of the disorder was not obvious based on the clinical tests. Other investigators have examined ACTH responses to CRH stimulation in adults with acquired hypocortisolism, contrasting the different results seen with hypothalamic or pituitary deficiencies. In most cases, the type of response seen is characteristic of the level of lesion. Minimal or undetectable basal and stimulated levels are generally associated with pituitary deficiencies, and high, normal or augmented stimulated levels are generally seen with hypothalamic deficiencies [Cantalamessa et al., 1990; Schulte et al., 1984; Tsukada et al., 1984]. In our cases, the laboratory studies, measuring random ACTH levels and the response to CRH stimulation did not unequivocally distinguish between ACTH and CRH deficiency. That the affected children have had measurable ACTH levels on some occasions suggests the defect is not in *POMC* transcription or processing to ACTH. In addition, the serum analysis showing normal levels of ACTH precursors present in the two affected children and their mother, although not definitive, is also consistent with a defect in CRH. The presence of these normal serum levels of precursors provides additional evidence that there is no significant abnormality of the POMC gene regarding transcription or post-translational processing, and that the gene is being expressed at a basal or constitutive rate. The mode of inheritance cannot be proven with this small family, but an autosomal recessively inherited single gene defect seems the most probable. Other possibilities include a new dominant mutation with germline mosaicism in one parent or expansion of a repeat element associated with a candidate gene in one parent. However, these alternative mechanisms seem less likely.

In our initial molecular studies, we examined restriction fragment lengths via Southern blotting. These results showed that there were no large deletions or rearrangements of the POMC gene. However, without further studies such as sequence analysis, these results provided limited information regarding ruling this candidate gene in or out as responsible for the cortisol insufficiency in these sibs.

The results of the microsatellite studies on the other hand, successfully identified one most likely candidate gene and excluded or made less likely the other three. The data obtained using markers near the POMC gene and both neuroendocrine convertase genes showed that the two affected sibs inherited different sets of paternal alleles, and in all three cases, one or the other of the unaffected sisters shared the same maternal and paternal haplotype as one of her affected sibs. The large recombination distances involved and the possibility of double recombination events between flanking markers means that in the absence of fully informative gene-specific markers, we cannot completely exclude *POMC, NEC-1,* or *NEC-2.* Nonetheless, the data allow us to place the highest priority on *CRH.* The data obtained from genotyping of markers on chromosome 8 strongly supports that the inheritance of hypocortisolism in this family has been inherited along with two specific maternal and paternal haplotypes containing the CRH gene. The information gained by these linkage studies provides high enough likelihood (8:1 odds in support of linkage) of a true linkage relationship to warrant proceeding with more detailed and time consuming studies such as sequence analysis. However, this technique is not error free. In addition to failure to appropriately select a group of candidate genes, provided also that all the candidates chosen have a known chromosomal location (for example, the linkage based location of the CRH receptor is not known; therefore, this gene cannot yet be screened for linkage using these methods), undetected crossovers may be present, especially in areas where there is minimal polymorphism of marker size. Clearly, though, the results obtained from linkage studies such as these provide the most rapid and useful initial information when searching for the genetic or biochemical basis of a clinical syndrome resulting from a single gene disorder. Microsatellite markers are now found at an average density of greater than 1 per cM across the human genome. Thus an exclusion analysis of particular candidate genes is a practical approach to prioritizing the candidates to study in greater detail. This type of analysis may be particularly useful in disorders of endocrine dysfunction which appear to segregate in mendelian fashion in even a small family. First, because the metabolic pathways for human biosynthesis and regulation are well understood for many processes providing an appropriate and adequate list of genes whose dysregulation could cause a particular disorder. And, second, since in disorders of high penetrance, sibships of even two can provide a sufficient material to exclude at least some genes. Thus it is especially important to identify other affected members in such families when an initial proband presents.

In this family, the relationship between ACTH deficiency and Chiari malformation is intriguing. Of course a common cause of the two abnormalities has not been established. However, their association in this family leads to speculation that the cause of corticotroph failure may have also produced the anatomical brain abnormality, perhaps in a manner similar to the SF-1 (steroidogenic factor-1) transcription factor. In the mouse, this factor is required for normal steroidogenesis, development of the gonads and adrenal glands, and development of the ventromedial hypothalamus [Ikeda et al., 1995].

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REFERENCES

Bartley JA, Patil S, Davenport S, Goldstein D, Pickens J (1986): Duchenne muscular dystrophy, glycerol kinase deficiency, and adrenal insufficiency associated with Xp21 interstitial deletion. J Pediatr 108:189–192.

Cantalamessa L, Catania A, Baldini M, Orsatti A, Motta P, Peracchi G (1990): CRH and lysine-vasopressin stimulation tests in the diagnosis of hypoadrenalism secondary to hypothalamic or pituitary disorders. Horm Metab Res 22:389–393.

Carey D (1985): Isolated ACTH deficiency in childhood: lack of response to corticotropin-releasing hormone alone and in combination with arginine vasopressin. J Pediatr 107:925–928.

Crosby SR, Stewart MF, Ratcliffe JG, White A (1988): Direct measurement of the precursors of adrenocorticotropin in human plasma by two-site immunoradiometric assay. J Clin Endocrinol 67:1272–1277.

Donohoue PA, Van Dop C, McLean RH, White PC, Jospe N, Migeon CJ (1986): Gene conversion in salt-losing congenital adrenal hyperplasia with absent complement C4B protein. J Clin Endocrin Metab 62:995–1002.

Feinberg AP, Vogelstein B (1984): A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Addendum. Analyt Biochem 137:266–267.

Gu J, Sadler L, Daiger S, Wells D, Wagner M (1993): Dinucleotide repeat polymorphism at the CRH gene. Hum Mol Genet 2:85.

Hansen JA, Fu SM, Antonelli P, Kamoun M, Hurley JN, Winchester RJ, Dupont B, Kunkel HG (1979): B-lymphocyte cell lines derived from HLA-D homozygous donors. Immunogenet 8:51–64.

Ichiba Y, Goto T (1983): Isolated corticotropin deficiency. Am J Dis Child 137:1202–1203.

Ikeda Y, Luo X, Abbud R, Nilson JH, Parker KL (1995): The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. Mol Endocrinol 9:478–486.

Jeffreys AJ, Wilson V, Thein SL (1985): Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73.

Kyllo JH,C ollins MM, Cuttler L, Rosenfield RL, Donohoue PA (1994): Gene studies in congenital isolated adrenocorticotropic hormone deficiency. Pediatr Res 35:102A.

Kyllo JH, Collins MM, Cuttler L, Rosenfield RL, Donohoue PA (1993): POMC gene studies in congenital isolated ACTH deficiency. Clin Res 43:665A.

Malpuech G, Vanlieferinghen P, Dechelotte P, Gaulme J, Labbe A, Guiot F (1988): Isolated familial adrenocorticotropin deficiency: prenatal diagnosis by maternal estriol assay. Am J Med Genet 29: 125–130.

Migeon CJ, Donohoue PA (1994): Adrenal disorders. In Kappy MS, Blizzard RM, Migeon CJ (eds): "The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence," 4th ED. Springfield: Charles C. Thomas, pp 717–856.

Murray JC, Duyk GM, Sheffield VC, Weber JL, Buetow KH, Weir RF (1993): Cooperative human linkage center report. 1:5–16.

Murray JC, Buetow KH, Weber JL, Ludwigsen S, Scherpbier-Heddema T, Manion F, Quillen J, Sheffield V, Sunden S, Duyk GM, Weissenbach J, Gyapay G, Dib C, Morrissette J, Lathrop GM, Vignal A, White R, Matsunami N, Gerken S, Melis R, Albertsen H, Plaetke R, Odelberg S, Ward D, Dausset J, Cohen D, Cann H (1994): A comprehensive human linkage map with centimorgan density. Science 265:2049–2054.

Muscatelli F, Strom T, Walker AP, Zanaria E, Recan D, Meindl A, Bardoni B, Guioll S, Zehetner G, Rabi W, Schwarz HP, Kaplan J-C, Camerino G, Meitinger T, Monaco AP (1994): Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. Nature 372:672–676.

NIH/CEPH Collaborative Mapping Group (1992): A comprehensive linkage map of the human genome. Science 258:67–86.

Nussey SS, Soo SC, Gibson S, Gout I, White A, Bain M, Johnston AP (1993): Isolated congenital ACTH deficiency: A cleavage enzyme defect? Clin Endocrinol 39:381–385.

Seo TS, Hanabusa T, Ohagi S, Steiner DF, Bell GI (1993): Dinucleotide repeat polymorphism in the NEC2 gene. Hum Mol Genet 2: 1983.

Schulte HM, Chrousos GP, Avgerinos P, Oldfield EH, Gold PW, Cutler Jr GB, Loriaux DL (1984): The corticotropin-releasing hormone stimulation test: A possible aid in the evaluation of patients with adrenal insufficiency. J Clin Endocrinol Metab 58:1064–1067.

Tsukada T, Nakai Y, Koh T, Tsujii S, Inada M, Nishikawa M, Shinoda H, Kawai I, Takezawa N, Imura H (1984): Plasma adrenocorticotropin and cortisol responses to ovine corticotropin-releasing factor in patients with adrenocortical insufficiency due to hypothalamic and pituitary disorders. J Clin Endocrinol Metab 58: 758–760.

Weber JL (1990): Informativeness of human $(dC\text{-}dA)_n$–$(dG\text{-}dT)_n$ polymorphisms. Genomics 7:524–530.

Zhou A, Bloomquist BT, Mains RE (1993): The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in strict temporal order during proopiomelanocortin biosynthetic processing. J Biol Chem 268:1763–1769.